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Evolutionary aspects of urea utilization by fungi

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Abstract
The higher fungi exhibit a dichotomy with regard to urea utilization. The hemiascomycetes use urea amidolyase (DUR1,2), whereas all other higher fungi use the nickel-containing urease. Urea amidolyase is an energy-dependent biotin-containing enzyme. It likely arose before the Euascomycete/Hemiascomycete divergence c. 350 million years ago by insertion of an unknown gene into one copy of a duplicated methylcrotonyl CoA carboxylase (MccA). The dichotomy between urease and urea amidolyase coincides precisely with that for the Ni/Co transporter (Nic1p), which is present in the higher fungi that use urease and is absent in those that do not. We suggest that the selective advantage for urea amidolyase is that it allowed the hemiascomycetes to jettison all Ni2+- and Co2+-dependent metabolisms and thus to have two fewer transition metals whose concentrations need to be regulated. Also, the absence of MccA in the hemiascomycetes coincides with and may explain their production of fusel alcohols.

Introduction
We have long been interested in the role of nitrogen sources in regulating fungal dimorphism. For instance, the growth morphology of Ceratocystis ulmi and Trigonopsis variabilis could be modulated by the source of nitrogen. For C. ulmi, the cells grew as yeasts with proline and as hyphae with ammonia, arginine, and most other nitrogen sources (Kulkarni & Nickerson, 1981), while for T. variabilis, the cells grew as budding yeasts with ammonium sulfate and as triangles with methionine (Sentheshanmuganathan & Nickerson, 1962). One nitrogen source that has been understudied in Candida albicans is urea. This inattention likely derives from numerous reports that C. albicans lacks urease (Odds, 1988), even though Dastidar et al. (1967) reported that urease in general is jettisoned (Nickerson, 1981), while for T. variabilis, the cells grew as budding yeasts with ammonium sulfate and as triangles with methionine (Sentheshanmuganathan & Nickerson, 1962). One nitrogen source that has been understudied in Candida albicans is urea. This inattention likely derives from numerous reports that C. albicans lacks urease (Odds, 1988), even though Dastidar et al. (1967) reported that most strains of C. albicans grew well with urea as the sole source of nitrogen. A partial resolution of this impasse derives from the fact that C. albicans uses urea amidolyase to hydrolyze urea (Ashok et al., 2009). The enzyme urea amidolyase, encoded by DUR1,2 (Degradation of URea), was first characterized in the yeast Candida utilis (Roon & Levenberg, 1972). This cytoplasmic, biotin-dependent enzyme (Roon et al., 1972) consists of a single protein chain with domains for both urea carboxylase and allophanate hydrolase activity (Cooper et al., 1980).

Distribution of urease and urea amidolyase in the fungi
To identify which fungi have urease and which have urea amidolyase, we examined 22 available fungal genomes spanning the Ascomycetes and Basidiomycetes for the presence of the respective genes. There is a dichotomy. Urease (Fig. 1a in red) was found in all of the fungi, except for members of the hemiascomycetes (Fig. 1a in black). The hemiascomycetes are those Ascomycetes that do not form fruiting bodies. The results are consistent with loss of the
urease gene sometime before the Euascomycete–Hemi-
ascomycete divergence c. 350 million years ago (Galagan et al., 2005).

The dual function urea amidolyase Dur1,2 occurs in two
sizes: the longer of c. 1800 aa (Fig. 1b in red) has a
1800-1200 aa ‘amidase’ domain fused at the N-terminus, whereas the
shorter of c. 1200 aa (Fig. 1b in orange) does not. The longer
DUR1,2 is present in all hemiascomycetes examined, includ-
ing C. albicans, whereas both the longer and the shorter
versions are found in subsets of the euascomycetes (Fig. 1b).

One explanation is that DUR1,2 arose via duplication of the
gene for methylcrotonyl CoA carboxylase (MccA), mito-
chondrial biotin-containing MccA (c. 700 aa), followed by
fusion of one of the MccA genes with another still unidenti-
fied gene (c. 500 aa) to generate DUR1,2. In the process, the
allophanate hydrolyase domain (Dur2) was inserted into the
biotin-containing MccA. The Zygomycetes and Basidi-
mycetes have only MccA (Fig. 1b in green), while the Hemi-
ascomycetes are missing MccA (Fig. 1b). Split red-green and
orange-green labels (Fig. 1b) indicate euascomycete species
that contain both DUR1,2 and MccA. Schizosaccharomyces
pombe does not contain either DUR1,2 or MccA. DUR1,2
likely originated before the split between the euascomycetes
and hemiascomycetes and was subsequently lost from
several euascomycete lineages, although the possibility for
multiple independent origins cannot be eliminated.

There is ample precedent for metabolic dichotomies in
the fungi. For instance, in lysine biosynthesis (Vogel, 1965),
the two pathways for lysine biosynthesis are named after the
intermediates that are characteristic of the paths; α-amino-
adipic acid (AAA) and diaminopimelic acid (DAP). Eugle-
nacea and all of the fungi use the AAA pathway, whereas all
other lysine prototrophs use the DAP pathway. No inter-
mediates or enzymes are common to the two pathways
(Vogel, 1965).

The distinct phylogenetic trees for urease (Fig. 1a) and
urea amidolyase (Fig. 1b) raise three further questions
regarding how and why those changes occurred. The first
concerns why two plant pathogens, Gibberella zeae and
Magnaporthe grisea, retain both urease and urea amidolyase

Fig. 1. Phylogeny of fungal urease, urea amidolyase, (Dur1,2), and MccA. Sequences of fungal proteins were obtained from NCBI (http://www.ncbi.
nlm.nih.gov) and the Fungal Genome Initiative (http://www.broad.mit.edu/annotation/fgi/). All BLAST searches were conducted using default
parameters. MACVECTOR software (Oxford Molecular Sciences Inc., Hunt Valley, MD) was used for processing and analysis of sequences. The dendogram
was prepared in POWERPOINT and represents the current view of fungal phylogeny as presented by James et al. (2006). (a) The presence (red) or absence
(black) of urease homologues was identified using Cryptococcus neoformans URE1 (AF006062) as a query for BLAST searches. (b) The presence of full-
length Dur1,2 (i.e. 1800 aa) is indicated in red, intermediate-length Dur1,2 (i.e. 1200 aa) is indicated in orange, and MccA is indicated in green. Split
red-green and orange-green labels indicate species that contain both Dur1,2 and MccA, while black indicates that none of the above genes was
detected. Dur1,2 and MccA homologues were identified using Saccharomyces cerevisiae Dur 1, 2 (CAA85172) as a query for BLAST searches.
when plants recycle virtually all of their amino groups and thus do not excrete urea. The second concerns the energetics of biotinylated enzymes. Most eukaryotes have only four biotin-containing enzymes: pyruvate carboxylase, propionyl-CoA carboxylase, acetyl-CoA carboxylase, and MccA (Samols et al., 1988). Why do the hemiascomycetes use an energy-dependent, biotin-containing urea amidolyase system when the same overall reaction could be accomplished by the simpler urease? This question becomes even more germane when we consider that all strains of C. albicans are biotin auxotrophs (Odds, 1988), and it has long been known that two to four times as much biotin is required for the maximum growth of S. cerevisiae on urea, allantoic acid, or allantoin as the sole nitrogen sources (DiCarlo et al., 1953).

**Loss of Ni$^{2+}$/Co$^{2+}$ enzymes**

Our current thinking is that DUR1,2 allows the hemiascomycetes to retain urea as a nitrogen source while jettisoning their last Ni$^{2+}$/Co$^{2+}$-containing enzyme. As part of a comparative genomic analysis, Zhang et al. (2009) examined 63 fungal genomes for consensus/predictive sequences associated with Ni$^{2+}$/Co$^{2+}$ transporters and the use of nickel and cobalt by fungi. Among biometals, nickel and cobalt are considered together because they are used at particularly low levels and often share a transport system. For the cobalt-containing vitamin B$_{12}$, three coenzyme B$_{12}$-dependent enzymes, methionine synthase, methylmalonyl-CoA mutase, and the B$_{12}$-dependent ribonucleotide reductase, were not found in any of the 63 fungal genomes (Zhang et al., 2009). Thus, in silico analysis concluded that the higher fungi do not use Co$^{2+}$ or any coenzyme B$_{12}$-dependent enzymes. However, marine lower fungi belonging to the Phycomycetes may still have a functional requirement for cyanocobalamin and may exhibit B$_{12}$ deficiencies (Goldstein & Belsky, 1963).

The situation for nickel was more intriguing in that there was a precise dichotomy between the hemiascomycetes and the rest of the higher fungi. Zhang et al. (2009) screened for the nickel/cobalt transporter (Nic1p) and the Ni-dependent enzyme urease. Neither was present in any of the 24 hemiascomycete genomes examined, but both genes were present in all eight of the Basidiomycetes, both of the Schizosaccharomyces, and 28 of the 29 Euascomycetes (Zhang et al., 2009). The exception was *Aspergillus terreus* ATTC 20542. Urease was the only Ni-dependent protein identified, and the taxonomic distribution of the nickel transporter and urease coincided exactly with that shown in Fig. 1a.

Thus, the Hemiascomycetes do not have any Ni- or Co-dependent enzymes, thus avoiding the delicate balance of acquiring the necessary trace levels of Ni$^{2+}$ and Co$^{2+}$ without exceeding the threshold levels at which those transition metals become toxic. For instance, Mackay & Pateman (1980) described a mutant of *Aspergillus nidulans* for which, with urea as the sole nitrogen source, 0.1 mM Ni$^{2+}$ was required, but 1 mM Ni$^{2+}$ was toxic. Using DUR1,2 instead of urease, the Hemiascomycetes can eliminate Ni$^{2+}$/Co$^{2+}$ transporters and have two less essential transition metals. Also, humans do not utilize nickel for major metabolic processes, and nickel is generally viewed as a toxic or a carcinogenic metal (Dosanjh & Michel, 2006). Switching to urea amidolyase would allow hemiascomycetes such as *C. albicans* to achieve urea degradation and kidney colonization in a nickel-deficient human host.

**Fusel alcohols**

The hemiascomycetes as a group have replaced MccA with urea amidolyase. Are there any phenotypes or negative consequences associated with this swap? MccA (EC 6.4.1.4) catalyzes the ATP-dependent carboxylation of 3-methylcrotonyl CoA to form 3-methylglutaconyl CoA. It is certainly involved in leucine catabolism (Rodriguez et al. (2004), and the production of fusel alcohols by yeasts may be a consequence of the loss of MccA. Fusel alcohols are derived from amino acid catabolism via a pathway proposed by Ehrlich (1907). For amino acids assimilated by the Ehrlich pathway (valine, leucine, isoleucine, methionine, and phe- nylnalanine), after the initial transamination reaction, the resulting a-keto acids cannot be redirected into central metabolism and are instead decarboxylated, reduced, and excreted (Hazelwood et al., 2008). The suggestion that fusel alcohol production derives from the loss of MccA predicts that only the hemiascomycetes should be capable of fusel alcohol production. This prediction has been partially confirmed by Penalva and colleagues. They found that Δmcc strains of *A. nidulans* could not grow on leucine as the sole carbon source and accumulated 3-hydroxyisovaleric acid in the culture supernatants. Although MccA is associated primarily with leucine catabolism (Rodriguez et al., 2004), we note that Northern analysis of *A. nidulans* mycelia revealed that MccA and MccB transcription was elevated in media containing 30 mM of leucine, valine, isoleucine, methionine, or phenylalanine, but not in any of the other four amino acids tested (Rodriguez et al., 2004).

**Urea and pathogenicity**

Urea catabolism has relevance because urease is a virulence factor in at least two pathogenic fungi Cryptococcus neoformans (Cox et al., 2000) and *Coccidioides immitis* (Cole, 1997) and two bacteria *Helicobacter pylori* (Eaton et al., 1991) and *Proteus mirabilis* (Jones et al., 1990). Having cytoplasmic urea catabolism (urea amidolyase) allows urea-dependent signalling pathways related to fungal pathogenicity (Ghosh et al., 2009). We examined the role of arginine-induced germ tube formation in the escape of *C. albicans*
from murine macrophages (Ghosh et al., 2009). Our studies link the work of Lorenz et al. (2004), who showed that the genes for l-arginine biosynthesis were induced following internalization by macrophages, with that of Sims (1986) and Mühlenschlegel’s group (Klengel et al., 2005; Bahn & Mühlenschlegel, 2006), who showed that elevated CO2 triggers hyphal growth. We connected these two observations via the enzymes l-arginase (Car1p), which converts arginine to urea, and urea amidolyase (Dur1,2p), which produces CO2. At that time, we generated a dur1,2/dur1,2 mutant from a wild-type parent (A72) and then reconstituted it. The dur1,2/dur1,2 mutant was unable to grow on urea as the sole nitrogen source, stimulate germ tube formation in response to l-arginine or urea, or escape from the murine macrophage cell line RAW 264.7. These abilities were restored in the reconstituted strains (Ghosh et al., 2009). Finally, ongoing studies also show that DUR1,2 is a virulence factor for C. albicans (D. Navarathna & D. Roberts, unpublished data).

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